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Does carnivory pay off? Experiments on the effects of different types of diet on growth and development of *Bufo bufo* (Linnaeus, 1758) tadpoles and carry-over effects after metamorphosis

Lohnt es sich ein Raubtier zu sein? Untersuchungen über die Auswirkungen unterschiedlicher Ernährungsweisen auf Wachstum und Entwicklung von *Bufo bufo* (Linnaeus, 1758) Kaulquappen und Carry-over Effekte nach der Metamorphose

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Abstract

Natural diets of anuran larvae vary widely in their relative amounts of nutrients. The proportion of these ingested nutrients has significant influence on larval and post-metamorphic performance.

Here, we use the Common Toad to address the role of diet (exclusively carnivore, exclusively vegetarian and mixed) on growth and development of tadpoles and short-term carry-over effects on post-metamorphic animals. Larvae fed on an exclusively vegetarian diet performed better (faster growth and development) than larvae fed on exclusively carnivore and mixed diets. Larvae fed on the exclusively carnivore diet had the lowest performance. Regarding the carry-over effects of larval diets, although the body condition indices of the toadlets were similar in all treatments, there was a major difference in the survival rate. While toadlets, originating from larvae fed on a vegetarian diet, were more successful and had the lowest mortality, those fed on a carnivore diet had the highest mortality level. Our results suggest that a plant-based diet may contain all the necessary nutrients needed by *Bufo bufo* larvae. Furthermore, a diet based exclusively on food of animal origin might be detrimental for the larval performance and could have significant carry-over effects on the post-metamorphic animal.

Kurzfassung

Die natürliche Nahrungszusammensetzung von Kaulquappen variiert stark in ihrem Nährstoffgehalt. Der Anteil der aufgenommenen Nährstoffe hat einen signifikanten Einfluss auf die Larven- und Post-Metamorphose-Entwicklung.

Wir verwenden Erdkrötenlarven, um den Einfluss der Nahrungszusammensetzung (ausschließlich karnivor, ausschließlich vegetarisch und Mischkost) auf das Wachstum und die Entwicklung der Kaulquappen und deren Kurzzeiteffekte auf das postmetamorphe Tier zu untersuchen. Larven, die mit ausschließlich pflanzlicher Nahrung gefüttert wurden, zeigten eine bessere Entwicklung (schnelleres Wachstum und schnellere Differenzierung) als Larven, die ausschließlich karnivor oder mit Mischkost gefüttert wurden. Ausschließlich karnivor aufgezogene Larven lieferten die schlechtesten Ergebnisse. Trotz ähnlicher Kondition der Jungkröten in

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allen drei Ernährungsvarianten, wiesen die karnivor aufgezogenen Kröten die höchste Mortalität auf. Pflanzlich ernährte Larven hatten die höchsten Überlebensraten im Postmetamorphose-Stadium. Unsere Ergebnisse deuten darauf hin, dass eine pflanzliche Ernährung alle Nährstoffe enthalten könnte, die *Bufo bufo*-Larven benötigen. Eine Ernährungsweise, die ausschließlich auf Nahrungsmitteln tierischen Ursprungs basiert, könnte für das Gedeihen der Larven schädlich sein und könnte auch signifikante Auswirkungen auf das postmetamorphe Tier haben.

Key Words

Amphibia, herbivory, grazing, experimental ecology, permanent pond-breeder

Introduction

Anuran larvae are essential components of many fresh-water communities. Their presence in these ecosystems is predominantly seasonal, reaching high densities and biomass (Schiesari et al. 2009) and, thus, substantially altering the balance between demographic density and resource availability and quality (Kupferberg 1997; Altig et al. 2007; Connelly et al. 2008). This balance can further be altered by global climate change and its effect on aquatic ecosystems, adding its contribution to the alarming rate of amphibian decline (Stuart et al. 2004; Lips et al. 2005).

Although they are usually considered to be herbivorous, in reality, the feeding habits of the majority of amphibian species in their larval stage remain largely unknown (Altig et al. 2007; Wells 2007).

Tadpoles show great morphological diversity and inhabit a wide variety of microhabitats (Altig and Johnston 1989). They are also capable of high trophic adaptation and physiological plasticity (e.g. Horiuchi and Koshida 1989), allowing them to considerably expand their ability to use a large variety of resources in stressful conditions. The trophic plasticity of anuran larvae can be very dynamic, ranging from herbivory to carnivory and includes predation (Richmond 1947; Ritchie 1982; Petranka and Kennedy 1999) and cannibalism (Heyer et al. 1975; Petranka and Kennedy 1999), scavenging, oophagy (Heusser 1971; Banks and Beebee 1987; Petranka et al. 1994), coprophagy, filter-feeding and hindgut microbial fermentation (Viertel 1983; Pryor 2014).

High dietary protein levels can enhance development, growth and survival and can increase size at metamorphosis (Nathan and James 1972; Steinwascher and Travis 1983; Pandian and Marian 1985). Some species of carnivorous tadpoles grow faster than herbivorous conspecifics (Crump 1990; Heinen and Abdella 2005). Furthermore, the capacity to shift towards a carnivore diet can be an especially important adaptive mechanism in cases of food depletion, allowing a substantial expansion of the trophic niche and hence allowing tadpoles to grow and develop till they reach the minimum developmental stage for triggering the metamorphosis (Wilbur and Collins 1973; Morey and Reznick 2000).

However, the mechanisms by which tadpoles process their diets and the nutritional importance of various food items are still largely under-investigated and remain unknown (Pryor 2014). Ingested and assimilated materials may differ substantially and might not necessarily reflect the trophic status of the animal (Altig et al. 2007). As a consequence, feeding tadpoles with various types of exclusive and mixed food can be a lucrative means of investigation on this subject.

Additionally, while there are a number of studies that indicate strong correlation between larval conditions and adult performance (Berven 1990; Goater 1994; Pechmann 1994), there is a lack of information on carry-over effects of the larval diet. Furthermore, experimental studies that incorporate both stages, pre-metamorphic and post-metamorphic, are also scarce.

Our study approached the issue of larval diet (exclusively vegetarian, exclusively carnivore and mixed) and its effects on larval growth and development, as well as potential carry-over effects of these three diets on the short term post-metamorphic performance of the Common Toad (*Bufo bufo*).

Based on what we know from literature, we formulated the following hypotheses:

1. A mixed diet will result in faster growth, larger size at metamorphosis and also higher survival rate, compared with the exclusive diets (vegetarian and carnivore).
2. Larval diet will have a carry-over effect. Specifically, toadlets resulting from larvae fed with mixed diet will have the best post-metamorphic performance.

We conclude by discussing the intricate issue of the diet during the larval stage of development in anurans and its significance for the larval and post-metamorphic animal.

Materials and methods

We used Common Toad tadpoles for testing our hypotheses. The Common Toad is a widespread species in Romania. It breeds in standing water, preferring permanent

ponds with no risk of desiccation (Jungfer 1943; Heusser 1958; Viertel 1978; Brady and Griffiths 2000).

The larva of *Bufo bufo* is a mid-water feeder with a preference for planktonic organisms (Harrison 1987). Savage (1952) analysed the gut content and found algae (*Scenedesmus*, *Pediastrum*), diatoms and also crustaceans (*Cladocera* and copepods), indicating that at least some part of the diet is of animal origin (Viertel 1981). Low clearance rate (time for food to pass through gut), short intestine and overall low profitability of the food ingested, suggest a low assimilation of food (Diaz-Paniagua 1989).

The preferred aquatic habitat is a eutrophic permanent pond (often with predatory fish e.g. Hartel et al. 2007) where food is usually abundant. In such conditions Common Toad tadpoles do not need high resource exploitation capabilities, like having a long intestine and a high clearance rate. Therefore, Common Toads metamorphose at a small size compared to adult body size (Diaz-Paniagua 1988, 1989).

The experiment

The experiment was conducted between March and June 2016 in the laboratory of the Vivarium of the Babes-Bolyai University, Cluj-Napoca, Romania and consisted of two components:

1. The larval experiment: the assessment of the feeding regime on tadpole growth and development.
2. The carry-over experiment: the short-term carry-over effect of the feeding regime on metamorphs.

Three entire Common Toad clutches were collected on 3 April 2016, from a large (ca. 3000 m²) permanent fishpond situated in the Faget forest, Cluj-Napoca, Cluj county, Romania (46°41'48,57''N 23°32'46,80''E (DMS), Someș-River Basin, elevation 682 m). They were kept for 11 days, each in a 20 litre container, until the hatching period was completed (14 April 2016). With the start of the feeding-larva stage (Smith-Gill and Berven 1979), we selected 120 healthy-looking tadpoles, 40 of each clutch, at ca. Gosner stage 25 (Gosner 1960) for the start of the experiment. The selected larvae were then randomly assigned to the three diet groups. All the other larvae were released into the original pond.

Three food treatments (hereafter referred as 'vegetarian diet', 'mixed diet' and 'carnivore diet'), with five replicates for each treatment were used. The replicates consisted of four-litre opaque containers (9×17 x 26 cm), each holding eight tadpoles in three litres of water (total of 40 larvae per treatment). This larval density corresponds to low densities in natural populations (Glennemeier and Denver 2002; Rot-Nikcevic et al. 2005). The water was kept at constant levels, at 20°C (+/-1) temperature and 12/12 hour light/dark photo period during the entire larval experiment, with light switching on and off at 9 am and 9 pm respectively. Light was generated by four 36 W fluorescent light tubes. Infra-red light was used during observations of night-time activity. Each container was provided with oxygen through electric air pumps. Over 90% of the water was changed every day with aged tap water, with occasional extra sanitation through siphoning, to clean organic materials.

The food for the 'vegetarian diet' group consisted of a mix of spirulina (Organic spirulina 500 mg tablets, protein 63.5%, carbohydrate 16.1%, fat 8.2%. origin: China), rabbit food (Versele Laga Cuni fit pure, protein 15%, carbohydrate 15% lipid 3%. origin: Hungary) and collard greens (protein 3%, carbohydrate 6%, lipid 1%. USDA), allowing them to selectively graze on the preferred food item. Spirulina was used in order to mimic the presence of high-protein microalgae species, e.g. from the genera *Chlorella, Scenedesmus, Anabaena, Aphanizomenon, Pediastrum,* identified in the original pond (Caraus 2012). The 'mixed diet' group was fed with vegetarian (see above) and carnivore diet, consisting of freeze-dried Tubifex worms (Bio-lio, 54% protein, 16% lipid, origin: Hungary) and freeze-dried Chironomidae larvae (Exo-ti-k, 30% protein, 10% lipid, origin: Romania). The carbohydrate levels for Tubifex worms and Chironomidae larvae were not provided by the manufacturers. However, according to literature, carbohydrate levels, expressed in % glycogen in the aerobic phase and at room temperature are ca. 5% for Tubifex worms and ca. 10% for Chironomidae larvae (Seuß et al. 1983; Hamburger et al. 1998). Diet types were alternated daily in the 'mixed diet' group. Finally, the 'carnivore diet' group was fed strictly with animal diet (see above). Tadpoles were fed ad libitum, every day, two hours after the daily water change.

The carry-over experiment targeted the growth and development of the toadlets. When the forelimbs emerged (ca. Gosner stage 42), the quantity of water in the holding containers was reduced to 2 litres and the containers were tilted so that a dry area formed at the raised end. Metamorphs were removed one-by-one, when they climbed out of the water at about Gosner stage 43 (starting date: 06 May 2016).

In order to provide a uniform diet, we used a single insect species as food item – *Acheta domesticus* (house cricket). Appropriate cricket size was judged as roughly the distance between the eyes of toads.

The crickets were raised on a combination of cat food, baby turtle food and spirulina in equal proportions with added turtle vitamin (Vita-Plus Vit A: 150.000 U.I.; Vit D3: 50.00. U.I.; Vit K: 25 mg). A small number of unconsumed crickets always remained in the enclosure until the following day proving that the toads were satiated.

Each surviving toadlet (n=106) was included in the experiment. The toadlets were housed in specially designed containers at a density of ca. 100 cm²/individual, keeping the same replications as for the larval experiment.

Enclosures were specially designed opaque plastic containers with textured sidewalls, to ensure better accuracy of the visual functions (Ewert et al. 2004). The

textures consisted in random patterns of yellow, black, green and opaque colours. As substrate, we used a wash-cloth-type material with good water retention capabilities (Craioveanu et al. 2017). A shelter was provided on the right side of the enclosure and consisted of two green artificial forest plant branches with seven leaves each (Exoterra forest plant) mimicking natural conditions (Craioveanu et al. 2017). Each container was cleaned once a week and dechlorinated tap water was used to maintain humidity. The initial environmental temperature was set to 20°C and was progressively raised over the period of seven weeks to 25°C to mimic natural conditions. The photoperiod was maintained the same as in the larval experiment (see above). The holding tanks' position was rotated/changed once a week.

In order to keep the individual density in the containers, dead toadlets were replaced with marked individuals (toe clipping) that were not used in the measurements.

All containers were held in a 7 x 5 m laboratory room with artificial ventilation. Lighting was provided by four 36 W fluorescent light tubes. Infra-red light was used during observations of night-time activity.

The carry-over experiment lasted for seven weeks and, at the end of the experiment, all the toadlets were released.

Measurements and analysis

All length measurements and developmental stage determinations were conducted on digital photographs using the free image analysis software IMAGEJ (http://imagej.nih.gov/ij). Photographs were taken using a Nikon D 3200 camera mounted 30 cm above the specimens.

In the larval experiment, we measured: total body length (snout to tail end in mm) and assessed developmental stages according to Gosner (1960) (multiple images from different angles). We also recorded the duration of the larval period (days) and mortality until and during metamorphosis. Due to the small size and fragility of the larvae at day 1 of the experiment (Gosner 25), the first measurements were made on day 7 (20 April 2016) with a total of three measurements performed weekly (days 7, 15 and 21 of the experiment).

The tadpoles were gently removed from their containers using a shallow net and placed in a scaled Petri dish containing tap water at a depth that approximately equalled the maximum dorso-ventral diameter of the tadpole (Davis et al. 2008). For the length measurements, care was taken to ensure that the animal was not moving during the photography session and the whole dorsal area was fully visible. After the photographs were taken, the tadpole was immediately returned to the tank.

We considered Gosner stage 43 as the time of metamorphosis. This was ecologically meaningful, as it marked the moment the metamorphosing animals first emerged from the water. We measured the body length (BL in mm) (the distance from the snout to the base of the remnant tail) (Altig 2007) daily, throughout the entire period of metamorphosis (06–14 May 2016; days 23–31 of the experiment).

In the carry-over experiment, we measured: snout to urostyle length (SUL in mm) and body mass (g). We used these measurements to calculate body condition (residual condition index) of individuals. The residual condition index was calculated as the value of the residuals resulting from the linear regression of body mass against the SUL (Băncilă et al. 2010) (R^2 varied between 0.78–0.84, computed in RSTUDIO version 1.1.463 (2016)). We also recorded mortality (% deaths) during metamorphosis and during the whole extent of the carry-over experiment.

To avoid excessive handling, for the metamorphosis and carry-over measurements we used the digital photographic technique combined with a scaled glass Petri dish containing 2-3 mm of water. We ensured that the animal was in a flat position, by replacing the lid (Antwis and Browne in http://www.amphibianark.org/). Immediately after the photograph was taken, the specimens were dried, by placing them briefly on a filter paper and weighed on an electronic scale to the nearest of 0.01g. The first measurement for the carry-over experiment took place 18 days after the last specimen left the water (2 June 2016; day 50 of the experiment) with a total of five measurements performed (days 50, 56, 64, 71 and 77 of the experiment).

For the larval experiment, we analysed the effect of 'Diet' (Fixed effect) on the development (Gosner stage) and growth (total body length, mm) of the Common Toad tadpoles with linear mixed models followed by Type I ANOVA, for each of the three measurements performed in the tadpole phase (we used car, MASS, nlme, lme4 packages in RSTUDIO Team (2016)). Subsequently, we tested for significant differences between the developmental progress of larvae fed with different diets, expressed in values of Gosner stages and total body length of larvae, at each measurement. To test for differences, we used analysis of variance (aov function in R) followed by Tukey HSD tests.

Data collected during metamorphosis were used to test the effect of 'Diet' (Fixed effect) on the time until metamorphosis with linear mixed models followed by Type I ANOVA (we used car, MASS, nlme, lme4 packages in RSTUDIO Team (2016)). We checked for differences in time until metamorphosis (number of days between the start of the experiment and metamorphosis), mortality during metamorphosis and BL at metamorphosis, in the three treatments. We tested for differences in mortality and BL with the Mann-Whitney U-test and analysis of variance followed by Tukey HSD tests, respectively.

For the carry-over experiment, we tested the effect of larval diet ('diet' = fixed effect) and period ('period' = number of days between metamorphosis and end of the experiment or death of the toadlets, fixed effect) on the body condition of the Common Toad metamorphs with linear mixed models followed by Type I ANOVA. Subsequently, we tested whether there were differences in residual BCI of the toads fed on different larval diets

with the help of a one-way ANOVA. We also analysed differences in the percentage of deaths occurring during metamorphosis and over seven weeks after metamorphosis with a one-way ANOVA followed by a Tukey HSD test. All analyses were performed in RSTUDIO version 1.1.463 (RSTUDIO Team 2016). Plots for Fig. 1 were drawn in RSTUDIO using ggplot2 package (RSTUDIO Team 2016).

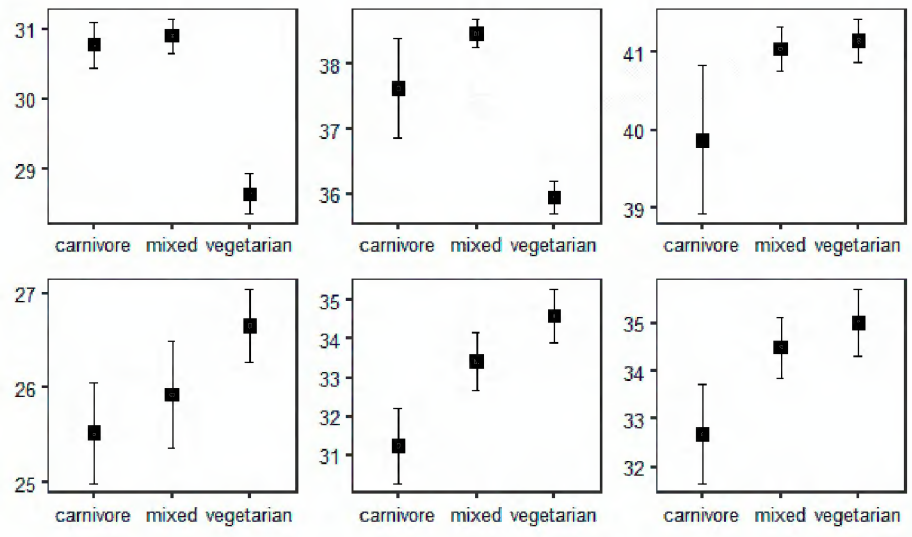

Figure 1. Mean developmental stage (Gosner) and mean total length of *Bufo bufo* larvae.
Mean developmental stage (Gosner) and mean total length in the three diet treatments at the three larval measurement days. Bars represent 95% confidence intervals.

Results

The descriptive statistics values for all measurements performed during the larval experiment, the metamorphosis period and during the carry-over experiment are summarised in Table1.

The effect of diet on larval development

The linear mixed models, followed by type I ANOVA, showed that the fixed effect "diet" had a strong effect on the development of larvae at all measurements (Table 2).

At the first measurement, we found significant differences between the Gosner stages of larvae fed with different diets ($F(2,117) = 79.88$, $P < 0.0001$). There was no significant difference between the Gosner stage of the larvae from the 'mixed' and 'carnivore' diet categories (Fig. 1, Tukey HSD test: $P = 0.81$). The larvae developing under 'vegetarian' diet had significantly lower Gosner stages than those from the 'mixed' (Tukey HSD test: $P < 0.0001$) and the 'carnivore' (Tukey HSD test: $P < 0.0001$) groups (Fig. 1).

At the second measurement, we found significant differences between the Gosner stages of larvae fed with different diets ($F(2,117) = 28.48$, $P < 0.0001$). We de-

Table 1. Descriptive statistics of the measurements performed in the larval experiment (Gosner stage, total body length), during metamorphosis (body length) and in the carry-over experiment (snout-urostyl length =SUL and body mass) on all *Bufo bufo* individuals.

	N	Mean	SD	Median	Min	Max	SE
Larval experiment							
day 7							
Gosner stage	120	30.11	1.36	30	26	32	0.12
Total body length (mm)	120	26.04	1.63	26.25	20.40	28.80	0.15
day 15							
Gosner stage	120	37.35	1.84	38	26	39	0.17
Total body length (mm)	120	33.09	2.89	33.05	21.80	39.3	0.26
day 21							
Gosner stage	120	40.69	1.94	41	26	43	0.18
Total body length (mm)	120	34.06	2.70	34.20	21.10	39.4	0.25
Metamorphosis							
Body length (mm)	114	12.73	1.02	12.80	10.20	15.70	0.10
Carry-over experiment							
Day 50							
SUL (mm)	106	14.57	1.91	14.70	10.90	20.50	0.19
Body mass (g)	106	0.25	0.10	0.24	0.06	0.55	0.01
Day 56							
SUL (mm)	83	15.49	2.14	15.50	9.60	20.40	0.24
Body mass (g)	83	0.27	0.12	0.25	0.11	0.73	0.01
Day 64							
SUL (mm)	62	16.45	1.86	16.40	12.60	20.30	0.24
Body mass (g)	62	0.33	0.12	0.31	0.10	0.66	0.02
Day 71							
SUL (mm)	62	17.40	2.15	17.65	12.00	22.00	0.27
Body mass (g)	62	0.41	0.13	0.41	0.18	0.72	0.02
Day 77							
SUL (mm)	62	18.79	2.18	18.95	13.50	23.50	0.28
Body mass (g)	62	0.55	0.17	0.53	0.21	0.90	0.02

Table 2. Effect of 'Diet' (Fixed effect) on the development (Gosner stage) and growth (total body length, mm) of the Common Toad tadpoles analysed with linear mixed models and Type I ANOVA.

	DF	*F*	*P*
Gosner stage			
Day 7			
Intercept	1,105	129888.25	<0.0001
Diet	2,12	76.37	<0.0001
Day 14			
Intercept	1,105	72206.88	<0.0001
Diet	2,12	28.48	<0.0001
Day 21			
Intercept	1,104	57066.82	<0.0001
Diet	2,13	5.78	0.01
Larval body length (mm)			
Day 7			
Intercept	1,105	32763.27	<0.0001
Diet	**2,12**	**5.32**	**0.02**
Day 14			
Intercept	1,105	16442.06	<0.0001
Diet	2,12	14.43	<0.0001
Day 21			
Intercept	1,104	14068.822	<0.0001
Diet	2,13	5.76	0.01

tected significant differences in the average Gosner stage for all three diet treatments (Fig. 1). The larvae, developing under the 'mixed' diet, had a higher average Gosner stage than those from the 'carnivore' (Tukey HSD test: $P = 0.04$) and 'vegetarian' diets (Tukey HSD test: $P < 0.0001$). The larvae from the 'vegetarian' diet had a significantly lower average Gosner stage than those from the 'carnivore' and 'mixed' diet categories (Tukey HSD test: $P < 0.001$ in both comparisons) (Fig. 1).

At the third measurement, we found significant differences between the Gosner stages of larvae fed with different diets ($F(2,117) = 5.78$, $P = 0.004$). The average Gosner stage of the larvae from the 'carnivore' diet was significantly lower than that of the larvae from the other two diet categories (Fig. 1, Tukey HSD test: 'mixed' $P = 0.02$, 'vegetarian' $P = 0.008$). There was no significant difference between the average Gosner stage of the 'mixed' and 'vegetarian' diets (Tukey HSD test: $P = 0.97$).

The 95% confidence interval (CI) of the average in the 'carnivore' category shows a constant increase in time, while in the other two diet categories, no trend in CIs was observable (Fig. 1).

The effect of diet on larval growth

The linear mixed models followed by type I ANOVA also showed that the fixed effect "diet" had a strong effect on the growth of larvae in all measurements (Table 2).

At the first measurement, on day 7, we found significant differences between the length of larvae fed with different diets ($F(2,117) = 5.33$, $P = 0.006$). The average length of the larvae from the 'carnivore' diet was the smallest and the 'vegetarian' diet was the largest (Fig. 1) (Tukey HSD test: $P = 0.005$), while the 'mixed' diet category was intermediate (not significantly different from the other two categories, $P > 0.05$ in both cases).

At the second measurement, on day 15, we found significant differences between the length of larvae fed with different diets ($F(2,117) = 17.71$, $P < 0.0001$). The body length of the larvae from the 'carnivore' diet category was significantly smaller than that from the 'mixed' (Tukey HSD test: $P = 0.0006$) and 'vegetarian' diet (Tukey HSD test: $P < 0.0001$). Furthermore, there was no significant difference between the 'mixed' and 'vegetarian' categories (Tukey HSD test: $P = 0.10$, Fig. 1).

At the third measurement, on day 21, we found significant differences between the length of larvae fed with different diets ($F(2,117) = 9.27$, $P = 0.0002$). We found the same pattern in average size distribution as in the second measurement, the larvae from the 'carnivore' diet category being significantly smaller than those from the 'mixed' (Tukey HSD test: $P = 0.005$) and 'vegetarian' ($z = 3.23$, $P = 0.0002$) categories, while no significant differences were found between the 'vegetarian' and 'mixed' groups (Tukey HSD test: $P = 0.62$) (Fig. 1).

The effect of larval diet on metamorphosis

Different diets did not have a significant effect on the time until metamorphosis (Gosner stage 43, Table 3). Mortality during metamorphosis was 0 for the individuals fed with a vegetarian diet, 0.05% (2 individuals) for the ones fed with a mixed diet and 0.1% (4 individuals) for those

fed on a carnivore diet. These mortality rates were not significantly different from each other (Mann-Whitney U-test: $P > 0.05$ in all comparisons).

The body length of *Bufo bufo* at metamorphosis varied significantly amongst the three treatments ($F(2,111) = 7.14$, $P = 0.001$). Body length of individuals fed with a carnivore diet was significantly lower than that of individuals fed with mixed (Tukey HSD test: $P = 0.005$) and vegetarian (Tukey HSD test: $P = 0.003$) diets, whereas those fed with mixed and vegetarian diets did not differ significantly from each other (Tukey HSD test: $P = 0.08$) (Fig. 2).

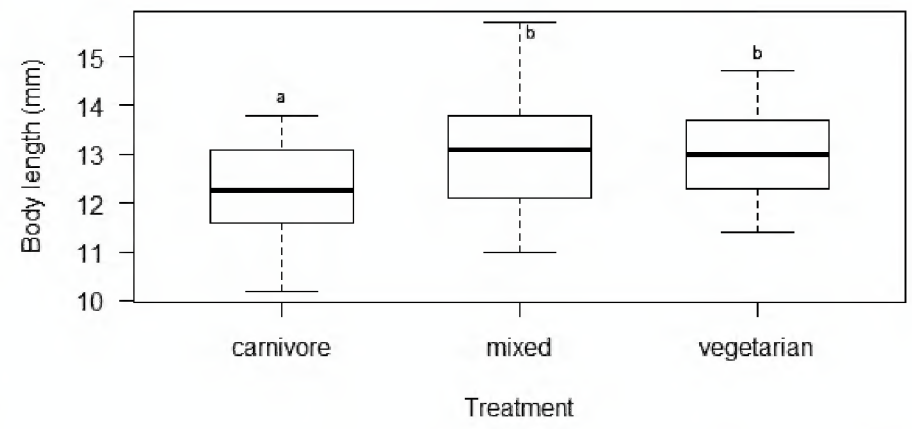

Figure 2. Body length of *Bufo bufo* at metamorphosis. Body length (mm) of *Bufo bufo* at metamorphosis (Gosner stage 43) for the three larval diets. Letters "a" and "b" indicate significant differences (one-way analysis of variance followed by Tukey HSD tests, $P < 0.05$).

Short term carry-over effect on metamorphs

We found a significant effect of 'period' on the metamorphs BCI but the effect of 'diet' was only marginally ($P < 0.1$) significant (Table 3).

At the first post-metamorphic measurement (day 50), we found significant differences between BCI of toads from different treatments ($F(2,103) = 5.48$, $P = 0.006$). The BCI of the toads from the 'vegetarian' group differed from those from the 'mixed' group (Tukey HSD test: $P = 0.004$).

At the second post-metamorphic measurement (day 56), we found no significant differences between BCI of toads from different treatments (Kruskal-Wallis rank sum test: $Chi^2 = 81.49$, DF = 80, $P = 0.43$).

At the third post-metamorphic measurement (day 64), we found significant differences between BCI of toads from different treatments ($F(2,59) = 6.31$, $P = 0.003$). Toads from the 'mixed' group had different BCI from those from the 'vegetarian' (Tukey HSD test: $P = 0.03$) and 'carnivore' groups (Tukey HSD test: $P = 0.004$).

At the fourth post-metamorphic measurement (day 71), we found no significant differences between BCI of toads from different treatments (Kruskal-Wallis rank sum test: $Chi^2 = 61.00$, DF = 61, $P = 0.48$).

At the fifth post-metamorphic measurement (day 77), we found no significant differences between BCI of toads from different treatments ($F(2,59) = 2.47$, $P = 0.09$).

Sixty-four days after the experiment started, mortality of the metamorphs in the 'vegetarian' diet reached 15%, in the 'mixed' diet 52.5% and in the 'carnivore' diet 77.5% (Fig. 3). Mortality between diets differed significantly after metamorphosis (ANOVA: $F(2,12) = 4.04$, $P = 0.046$). Mortality in the carnivore diet was significantly higher than that in the vegetarian diet (Tukey HSD test: $P = 0.04$). The other two diets did not differ significantly regarding metamorph mortality (Tukey HSD test: Pveg-mix = 0.23, Pmix-car = 0.55).

Discussion

In this study, we investigated life history responses of three types of larval diet: vegetarian, mixed and carnivore, containing varied proportions of proteins, lipids and carbohydrates on Common Toads.

The nutritional requirements of tadpoles are extremely diverse and incompletely understood (Alvarez and Nicieza 2002). What we know so far is that the relative amount of nutrients available to tadpoles (proteins, lipids and carbohydrates) can influence the thyroid hormone function, which, in turn, influences metamorphosis (Kupferberg 1997). Out of the three nutrient categories, dietary protein appears to be the most important at this stage. Through its stimulating effects on the thyroid function, it has an enhancing effect on differentiation and metamorphosis (Steinwascher and Travis 1983; Pandian and Marian 1985; Soamiarimampionona et al. 2015) and an inhibiting effect on growth (Etkin 1968, 1969). On the other

Table 3. Effect of 'Diet' (Fixed effect) on the time of metamorphosis and effect of 'Diet' and 'Period' (Fixed effects) on the body condition index of the Common Toad metamorphs analysed with linear mixed models and Type I ANOVA. The intercept and SD of the residual of 'Individual' and 'Replicate' are considered random effects.

	DF	F	P
Time until metamorphosis			
Intercept	1,110	33684.19	< 0.0001
Diet	2,110	1.44	0.24
BCI			
Intercept	1,364	0.002	0.95
Diet	2,364	2.39	0.09
Period	4,364	23.52	< 0.0001

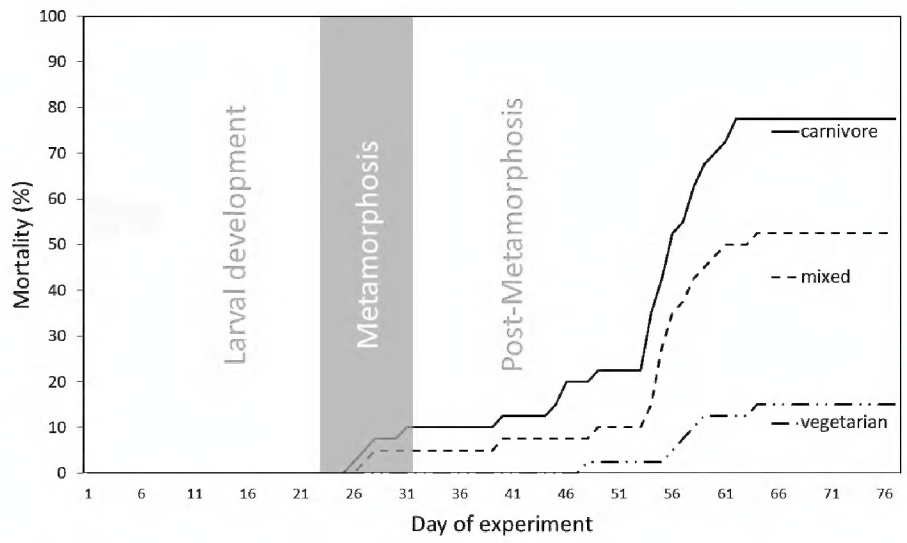

Figure 3. Mortality of *Bufo bufo*. Mortality (%) during larval development (day 1–23), metamorphosis (day 24–31) and post-metamorphic stage (day 32–77) in *Bufo bufo* for the three larval diets.

hand, a low protein content diet, combined with carbohydrates, promotes growth with slower development (Richter-Boix et al. 2007).

For permanent pond breeder anurans, such as *Bufo bufo*, the Wilbur-Collins model (Wilbur and Collins 1973; Brady and Griffiths 2000) predicts a variable larval developmental period needed to reach an optimal body size, in environments of variable suitability. The body size at metamorphosis will have a narrow range in this model. Therefore, we expected that, regardless of the diet type, the tadpoles participating in our study will metamorphose roughly at similar sizes but at variable time periods, depending on how satisfactory the diet was.

In contradiction to the above-mentioned model, in our experiment the sizes at metamorphosis differed significantly between the treatments, but the larval period was not significantly different. Additionally, we found a variation in the dynamics of the larval development and growth. The tadpoles, fed with an exclusively carnivore diet, accelerated their development at the beginning of the experiment to the detriment of growth. Meanwhile, tadpoles, fed with an exclusively vegetarian diet, had a slow development at the beginning, investing resources in increasing their body size.

In all measurements, the larvae, fed with vegetarian diet, were the largest, followed by those fed with a mixed diet. Larvae, fed on carnivore diet, had constantly smaller body sizes. All larvae had a 100% survival rate, regardless of the diet type (Fig. 3).

The first weeks after metamorphosis are critical for the toadlets. This is the period with the most rapid growth (Breckenridge and Tester 1961; Labanick and Schlueter 1976) and the highest mortality (Pechmann et al 1991). In the case of bufonid toads, it is even more critical, since they metamorphose at a very small size (Werner 1986). Therefore, it is likely that the most important carry-over effects will occur in this period. In our experiment, diet had no effect on the BCI of the toadlets, remaining similar throughout all the measurements. The main difference consisted in the survival rate, which decreased substantially after metamorphosis, compared to the larval period. We also recorded significant differences in the survival rates of toads fed on different larval diets: vegetarian 85%, mixed diet 47.5%, carnivore 22.5% (Fig. 3). However, by the third post-metamorphic measurement (32 days after the metamorphosis), the mortality stabilised (Fig. 3), possibly indicating that the carry-over effect of larval diet on toadlets survivability began to subside.

We cannot exactly identify the nutrient component or components that were responsible for our results. Since the vegetarian diet group clearly outperformed the other two, we can suggest that higher carbohydrate levels present in the vegetarian diet played a consistent role.

The protein levels were high throughout all the three diets. However, studies carried out on juvenile trout (Riley et al. 1993) demonstrated that the amino-acid composition of the protein also influences thyroid function. Accordingly, a high dose of animal protein in the form of collagen can lead to poor conversion in body protein, thus inhibiting growth and development. This depressing effect on the thyroid function could also explain the results of Coretti-Helfer (1976), where *Rana temporaria* tadpoles, fed with animal protein, were not able to finish their development and had 100% mortality as larvae. Since, in our experiment on *Bufo bufo*, the carnivore group also yielded the smallest individuals, we cannot exclude the possibility that high doses of animal protein may produce a similar reaction in other anuran species, with significant carry-over effects.

The same larval development period and the 100% survival rate in the larval stage, in all three diet treatments, are also unexpected. The larval diet containing exclusively food of animal origin had an evidently adverse effect on both tadpoles and toadlets. However, probably this effect is not part of the detrimental ecological conditions that trigger acceleration of the time to metamorphosis, even with high protein trophic background.

Digestive plasticity can also be a factor to take into consideration. Anurans like *Bufo bufo,* breeding in permanent ponds, with no danger of desiccation, can simply be less flexible than those breeding in temporary ponds, when it comes to broadening their nutrient resources.

All these considerations are supported by the results of our larval experiment, where the tested diet of animal origin had an inhibiting effect on growth and development, when compared with food of vegetal origin. Counter-intuitively, a mixed larval diet was also suboptimal compared with an exclusive vegetarian diet.

High mortality in the first part of the carry-over experiment (77.5%) and also the stabilising of the mortality after the first 3 weeks, suggest that the negative effect of food of animal origin in the larval period transcended metamorphosis and may have acted like a density-dependent regulation of population size. More precisely, in case of resource depletion, Common Toad tadpoles could, to some extent, complete their diet with food of animal origin, without significant carry-over effects. However, the

more food of animal origin present in the diet, the higher the cost paid by post-metamorphic animals, in terms of survival. As costly as it seems, this trade-off could also be useful as a limiting factor in case of alpine populations breeding in oligotrophic lakes.

Consequently, the starting hypotheses of this study were not confirmed. A mixed diet was not needed and had a limiting effect on the performance of the tadpoles and toadlets. Amongst the exclusive diets, the vegetarian diet provided the best performance in the larval stage and after metamorphosis, suggesting that it contained all the required essential nutrients. The diet of animal origin was accepted but poorly tolerated, generating overall low performance in the larval stage and high mortality after metamorphosis.

Many questions regarding the optimal diet for the anuran larvae remain to be answered in the future. Laboratory experiments give us the opportunity to test the effects of different food types on tadpoles and post-metamorphic animals. As amphibians increasingly face extinction, there is a need to improve the success rates of captive breeding and re-introduction programmes. Using non-threatened species as surrogate species in order to develop husbandry protocols before collecting a target high-risk species can be an important shortcut in achieving conservation objectives. In this particular case, we consider *Bufo bufo* to be a possible surrogate for closely related declining species of the *Bufo bufo*-complex (e.g. *Bufo verrucosissimus* – IUCN near threatened, *Bufo eichwaldi* – IUCN vulnerable). The Common Toad shares a strong phylogenetical and taxonomical relationship with these species (García-Porta et al. 2012) and also a high ecological similarity (Mertens and Wermuth 1960).

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